Nitric oxide enhances experimental wound healing in diabetes

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Background: Diabetes is characterized by a nitric oxide deficiency at the wound site. This study investigated whether exogenous nitric oxide supplementation with the nitric oxide donor molsidomine (N-ethoxycarbomyl-3-morpholinyl-sidnonimine) could reverse the impaired healing in diabetes.

Methods: Wound healing was studied by creating a dorsal skin incision with subcutaneous polyvinyl alcohol sponge implantation in diabetic and non-diabetic rats. Half of each group was treated with molsidomine. Collagen metabolism was assessed by wound breaking strength, hydroxyproline (OHP) content, RNA expression for collagen type I and III, and matrix metalloproteinase (MMP) 2 activity in wound sponges. Wound fluid, plasma and urinary nitric oxide metabolite levels, and the number of inflammatory cells were assessed.

Results: OHP content and wound breaking strength were significantly increased by molsidomine. MMP-2 activity in wound fluid was decreased in diabetes and upregulated by nitric oxide donors. The impaired inflammatory reaction in diabetes was unaffected by nitric oxide donor treatment and ex vivo nitric oxide synthesis was no different between wound macrophages from control and diabetic animals, suggesting that the nitric oxide deficiency in the wound is due to a smaller inflammatory reaction in diabetes.

Conclusion: The nitric oxide donor molsidomine can at least partially reverse impaired healing associated with diabetes.

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Introduction

The problem of impaired healing in diabetes is still not understood. However, all stages of the complex wound healing cascade, including inflammation, proliferation, angiogenesis and matrix formation, are impaired. Decreased chemotaxis, phagocytosis, bacterial killing and antioxidant levels during the early phase of repair have all been related to impaired healing in diabetes. Growth factor depletion, increased glucocorticoid levels, decreased cell proliferation and upregulation of apoptosis characterize the later phases of healing in diabetics, resulting in poorer granulation tissue formation. Decreased α-actin smooth muscle expression and an excess of adipose tissue may also affect healing in diabetics. Local administration of growth factors has been explored as a potential clinical treatment, but the results have been mostly disappointing. Restoration of blood glucose by insulin partially reverses some of the diabetes-induced wound healing deficiencies such as restoration of insulin-like growth factor levels, and improvement of bacterial killing and collagen metabolism. Few therapeutic alternatives are therefore available to reverse impaired healing in diabetes.

The role of nitric oxide as a mediator in wound healing has been elucidated recently. Administration of supplemental L-arginine, the sole substrate for inducible nitric oxide synthase (iNOS), improves wound healing, whereas lack of dietary L-arginine delays wound healing in experimental models. iNOS knockout mice. Nitric oxide donors increase collagen synthesis in fibroblasts in vitro by an unknown mechanism and accelerate wound closure when applied topically to excisional wounds. Inhibition of iNOS impairs incisional and excisional healing. Equivocal results have been obtained, however, from wound healing experiments in iNOS knockout mice. Although nitric oxide regulates important functions of wound healing, such as cellular proliferation, angiogenesis, chemotaxis and epithelialization, the exact mechanism of action remains unclear.

Based on previous findings that diabetes is characterized by reduced nitric oxide levels in the wound environment, the present study investigated whether exogenous nitric
Nitric oxide can restore the impaired wound healing in experimental diabetes, using molsidomine, a widely used antianginal drug which spontaneously releases nitric oxide.

**Materials and methods**

**Materials**

Molsidomine (N-ethoxycarbonyl-3-morpholinyl-sidnonine) was purchased from Sigma (St Louis, Missouri, USA) and dissolved in ethanol/water (1/99 v/v).

**Animals and induction of diabetes**

The study was approved by the animal care committee. Male Sprague–Dawley rats (Harlan-Sprague Dawley, Indianapolis, Indiana, USA) were allowed to acclimatize for 1 week and were offered food and water *ad libitum* throughout the study. Nine days before wounding, 22 rats were injected intraperitoneally with streptozotocin (70 mg per kg body-weight in citrate buffer 0.1 mol/l, pH 4.5) to induce diabetes. Eighteen control rats were injected with citrate buffer only. Induction of diabetes (blood glucose greater 250 mg/dl) was confirmed by venous glucose measurement. Animals not achieving the diabetic state after 24 h were reinjected with streptozotocin and then excluded from the study if the glucose concentration was not greater than 250 mg/dl.

Half the control and half the diabetic group were treated with molsidomine (1 mg per kg body-weight per day), and the other half received ethanol/water (1/99 v/v) only. The treatment was administered by gavage three times daily in an approximate volume of 2 ml per gavage and started on the day of wounding. All animals were weighed daily and monitored for side-effects.

**Wounding**

Rats underwent a 7-cm dorsal skin incision and bilateral subcutaneous implantation of polyvinyl alcohol (PVA) sponges (M-PACT, Eudora, Kansas, USA) under intraperitoneal pentobarbital anaesthesia. The incision was closed with surgical staples. Ten days after wounding the rats were killed, blood was obtained by cardiac puncture and the following variables were analysed.

**Blood chemistry**

Blood glucose, protein, albumin, blood urea nitrogen and creatinine were measured in venous blood.

**Urinary nitrate and nitrite**

Four to five rats per group were kept in metabolic cages and urine was collected from postoperative day 3 to day 4. Samples were acidified to prevent bacterial overgrowth. Urine samples were analysed for nitrite and nitric oxide metabolite (NO₃) content as described below. Results are expressed as micromoles of NO₃ per day per rat based on total volume of urine excreted.

**Breaking strength**

The dorsal skin pelt containing the incision was excised widely and cut by a multibladed guillotine into equal strips, each containing a segment of the healing scar. Immediately after death, four strips from each animal were analysed for wound breaking strength (WBS) using a constant-speed tensiometer.

**Hydroxyproline content of sponges**

The two most cephaled preweighed sponges were cleared of surrounding granulation tissue and used to measure hydroxyproline (OHP) content (micrograms per 100 mg sponge), an index of collagen deposition, as described previously.

**Wound fluid analysis**

Sponges were cleared of surrounding granulation tissue, and wound fluid was harvested and prepared for NO₃ measurement as described previously. Arginase activity was quantified spectrophotometrically by urea formation under optimal conditions and expressed as nmoles of urea per minute per milligram protein.

**Extraction of wound cellular infiltrate**

Four sponges per animal were used. The squeezed sponges were minced with iris scissors in complete Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life technologies, Grand Island, New York, USA) containing glutamine, penicillin, streptomycin, fungizone and 1 per cent albumin. The cell suspension was passed through an 80 and 100 stainless steel mesh and combined with the cell pellet from the wound fluid preparation which was retrieved after the first spin (400g for 10 min). After lysis of red blood cells in ammonium chloride buffer, the cells were washed and counted using a Neubauer chamber. Viability was checked by trypan blue exclusion.
In vitro nitrite synthesis by wound macrophages

Wound cells (1.5 × 10^6) were seeded into 24-well plates (Costar, Cambridge, Massachusetts, USA) in phenol red-free DMEM containing 1 per cent albumin and L-arginine 1 mmol/l, and cultured for 24 h. A macrophage-enriched wound cell population was obtained by vigorously washing off non-adherent cells 2 h after plating. Half of the macrophage-enriched wound cells thus obtained were stimulated with lipopolysaccharide 1 μg/ml and interferon-γ 10 units/ml (Boehringer Mannheim, Indianapolis, Indiana, USA). After 24 h the medium was harvested and the cells were lysed for DNA analysis by the Hoechst Dye technique as described previously27. Nitrite synthesis values were normalized for DNA concentration. Determination of nitrite was performed using the Griess reagent28.

Northern blot analysis

One sponge per animal was used to extract RNA using TRIzol reagent (Gibco Life Technologies) following the manufacturer’s instructions. Some 10–15 μg RNA was separated on 1%–2% agarose gels, transferred on to nylon membranes (Amersham Life Sciences, Arlington Heights, Illinois, USA) and cross-linked. After prehybridization using salmon sperm DNA, the blots were hybridized at 42°C using mouse collagen types I and III (gift from Dr Ksander, Celltrix, Palo Alto, California, USA) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes (generated by polymerase chain reaction). After washing, the blots were autoradiographed. Blots were reprobed with GAPDH to control for equal loading. Densitometry was performed using Image Quant™ software (Molecular Dynamics Image Quant™, Santa Barbara, California, USA). Results are expressed as the ratio of the density for collagen to that for GAPDH; controls were set at 1.

Zymography

The gelatinase activity of equal amounts of wound fluid proteins (Table 1) was measured following standard non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5 per cent acrylamide gel containing 0.1 per cent gelatin type A as substrate (Fisher, Hampton, New Hampshire, USA), as described previously29. The gel was incubated overnight at 37°C in Tris-hydrochloride 50 mmol/l (pH 7.6), sodium chloride 0.2 mol/l, calcium chloride 5 mmol/l and 0.02 per cent (w/v) Brij35, stained in 0.25 per cent coomassie blue and destained.

Table 1 Blood chemistry and wound fluid protein levels

<table>
<thead>
<tr>
<th></th>
<th>Control + saline</th>
<th>Control + molsidomine</th>
<th>Diabetic + saline</th>
<th>Diabetic + molsidomine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum protein (g/l)</td>
<td>48-1(4-0)</td>
<td>49-3(4-3)</td>
<td>45-0(0-9)</td>
<td>46-1(1-2)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4-7(0-4)</td>
<td>4-3(0-2)</td>
<td>28-5(1-4)*</td>
<td>26-4(1-2)*</td>
</tr>
<tr>
<td>Blood urea nitrogen (mmol/l)</td>
<td>9-3(1-2)</td>
<td>8-1(0-3)</td>
<td>12-8(0-9)*</td>
<td>13-3(1-3)*</td>
</tr>
<tr>
<td>Wound fluid protein (g/l)</td>
<td>65-1(0-6)</td>
<td>68-8(1-1)</td>
<td>51-9(2-1)*</td>
<td>53-3(2-1)*</td>
</tr>
</tbody>
</table>

Values are mean(s.e.m.). *P < 0.05 versus control + saline (analysis of variance with Fisher’s post-hoc test)

Results

Four of the 22 streptozotocin-injected rats did not become diabetic and were excluded. Two other diabetic animals were excluded because gastric gavage was not consistently possible. At the end of the study 18 control and 16 diabetic rats could be evaluated.

Treatment with molsidomine was well tolerated as demonstrated by the equal weight gain in the non-diabetic groups. Treatment with molsidomine resulted in significantly less weight loss in the diabetic rats (± 4(12) g for molsidomine-treated diabetic rats versus − 21(7) g for saline-treated diabetic rats; P < 0.05).

Blood glucose values were significantly increased in the diabetic rats, but were not affected by molsidomine treatment (Table 1). Wound fluid protein levels, a reflection of the degree of the inflammatory response, were significantly lower in diabetic rats. BUN was also higher in the diabetic animals, most probably indicative of the increased protein catabolism. Molsidomine had no effect on any of these variables.

Molsidomine treatment significantly increased the fresh WBS in the diabetic group but did not affect WBS in the control group. This was paralleled by a significant increase in OHP content with molsidomine in the subcutaneously
implanted PVA sponges in both control and diabetic animals (Table 2).

Diabetic rats had significantly less NOx in wound fluid than controls indicating a nitric oxide-deficient state (Fig. 1). Molsidomine treatment increased NOx excretion in urine, but did not affect NOx levels in wound fluid or plasma.

Zymograms of wound fluid, used as an indicator of gelatinase activity, showed significantly less activity in diabetes of an approximately 60-kDa protein, corresponding to MMP-2. Molsidomine treatment specifically increased this gelatinase activity in zymograms of control as well as diabetic rats (Fig. 2).

Northern blot analysis of collagen type I and III gene expression from the sponges showed a 25 per cent decrease in gene expression in the treatment groups compared with controls. For densitometry the northern blots were normalized against GAPDH and the non-treated groups were set at 1. Collagen type III gene expression was affected in the same way as collagen type I expression (data not shown).

The inflammatory response to wounding, represented by the number of cells isolated per sponge, was significantly decreased in diabetic rats compared with controls (approximately 40 per cent) but was unaffected by molsidomine treatment (Table 3). This was confirmed indirectly by a lowering of RNA per sponge from the diabetic rats as well as a lower protein concentration in wound fluid. The ex vivo nitric oxide formation by wound-derived macrophages was approximately equal in all groups as was the increase in nitric oxide synthesis following exposure to lipopolysaccharide and interferon-γ (Fig. 4).

Arginase activity in wound fluid, representing the alternative pathway of arginine metabolism in wounds, was significantly lower in diabetic wound fluid (Fig. 5).

**Discussion**

The present study confirms that diabetes is characterized by a nitric oxide-deficient state accompanied by decreased WBS, collagen deposition and a severely impaired inflammatory response. The nitric oxide donor molsidomine

| Table 2 Wound breaking strength and hydroxyproline content of polyvinyl alcohol sponges |
|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
|                                | Control + saline | Control + molsidomine | Diabetic + saline | Diabetic + molsidomine |
| OHP content in PVA sponges (µg per 100 mg sponge) | 1311(105) | 1645(82)* | 555(96)* | 902(131)* |
| Wound breaking strength (g) | 409(33) | 417(25) | 184(12)* | 258(33)* |

Values are mean(s.e.m.). OHP, hydroxyproline; PVA, polyvinyl alcohol. *P < 0.05 versus control + saline, †P < 0.05 versus diabetic + saline (analysis of variance with Fisher’s post-hoc test)
represents a potential treatment for acutely impaired healing in diabetes because it increased wound breaking strength by increasing the OHP content of the PVA sponges, a marker of collagen deposition. This nitric oxide donor did not affect collagen gene transcription\(^{16}\) so a post-transcriptional regulatory mechanism has to be assumed.

The data further show that hyperglycaemia impairs the inflammatory response during wound healing as demonstrated by the decreased number of wound inflammatory cells in diabetes. Hyperglycaemia and nitric oxide are both known to impair the chemotactic response, but in this study cellular infiltration was unaffected by nitric oxide donor treatment (Table 3). The reduced inflammatory response involves reduced macrophage infiltration, a process essential for wound healing\(^{30}\). As the in vitro nitric oxide synthesis of wound-derived macrophages was no different between control and diabetic rats, the difference in accumulation of nitric oxide metabolites in wound fluid was rather due to the significantly smaller number of infiltrating cells. There was also no difference in nitric oxide production after in vitro stimulation of wound-derived macrophages with the cytokine mix. This suggests that inflammatory cells in the wound milieu are similarly sensitive to nitric oxide stimulation, although it is not certain that the ex vivo culture of macrophages fully reflects in vivo regulation. InOS synthesis in both control and diabetic macrophages was not fully stimulated indicating that the level of nitric oxide stimulation during wound healing is tightly controlled physiologically.

Nitric oxide donor treatment does not completely restore nitric oxide levels in wound fluid or plasma (Fig. 1). The effect of nitric oxide donor administration may be dependent on a threshold rather than a dose. So far, no study has demonstrated a correlation between levels of nitric oxide in wounds and outcome. This corresponds with a previously published study in which administration of nitric oxide donors to fibroblasts induced a significant increase in collagen synthesis within a small range\(^{16}\) or with the long-lasting effect of a single transfection of iNOS complementary DNA in iNOS knockout mice\(^{25}\). It is conceivable that diabetic wounds are more susceptible to nitric oxide donor treatment since the wound is deficient in nitric oxide. The effect in non-diabetic rats might have been more pronounced if a higher dose of molsidomine was used. The dose chosen was rather low to avoid interference by the vasodilator effect of the nitric oxide donor. Molsidomine has an affinity for skin tissue\(^{31}\) which might explain why it affects dermal wound healing with little systemic effect.

Interestingly there was significantly lower arginase activity in wound fluid from diabetic animals. Arginase is released into wound fluid by macrophage cell death\(^{32}\).

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**Table 3** Inflammatory response measured as the number of infiltrating cells per sponge and cell viability

<table>
<thead>
<tr>
<th></th>
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<th>Diabetic + saline</th>
<th>Diabetic + molsidomine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells per sponge</td>
<td>4.19(0.39)</td>
<td>4.41(0.42)</td>
<td>2.41(0.45)*</td>
<td>2.93(0.39)*</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>54.8(2.3)</td>
<td>53.5(3.4)</td>
<td>54.2(2.6)</td>
<td>50.4(2.6)</td>
</tr>
</tbody>
</table>

Values are mean(s.e.m.). *\(P < 0.05\) versus control + saline (analysis of variance with Fisher’s post-hoc test)
However, fibroblasts also contribute to wound arginase activity\textsuperscript{33}. The reduced arginase activity seems to be specific to the wound since liver arginase is upregulated in diabetes\textsuperscript{34}. As arginase activity was unaffected by treatment with molsidomine it seems unlikely that this alternative pathway of nitric oxide metabolism is responsible for the effect in the treatment group.

Fig. 3 \textit{a} Representative Northern blot showing collagen type I gene expression. RNA was extracted from sponges and analysed for collagen type I expression using specific DNA probes. \textit{b} For analysis, densitometric values in control groups were set at 1 and those in molsidomine-treated groups were given relative to this. Molsidomine treatment decreased collagen type I gene expression in both groups. The same effect was seen for collagen type III (data not shown). GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Fig. 4 Mean(s.e.m.) nitrite accumulation over 24 h in unstimulated wound macrophages or macrophages stimulated with cytomix (lipopolysaccharide–interferon-\(\gamma\)). Control and diabetic macrophages produced similar amounts of nitrite in culture. Stimulation with cytomix increased nitrite synthesis to the same extent in both groups

Fig. 5 Mean(s.e.m.) arginase activity in wound fluid. Arginase activity was significantly reduced in diabetic animals. The effect of impaired cellular infiltration in diabetic sponges was taken into account by expressing enzymatic activity with respect to protein content in wound fluid, so the reduced activity was due to a net reduction in activity. *\(P < 0.05\) versus control + saline, †\(P < 0.05\) versus control + molsidomine (analysis of variance with Fisher’s post-hoc test)
WBS, an indicator of the amount and quality of newly deposited collagen at the wound site, was significantly increased by nitric oxide donor treatment in diabetes. This effect was accompanied by increased OHP in the sponges indicating that the nitric oxide donor is probably acting by increasing new collagen formation. Northern blotting, however, revealed no effect of nitric oxide treatment on collagen gene expression. The wound fluid of diabetic animals had significantly less MMP-2 activity than controls, which was significantly increased by nitric oxide donor treatment (Fig. 2). MMP-2 originates primarily from fibroblasts and is involved in remodelling of wound healing. Upregulation of MMP-2 might reflect increased matrix remodelling by molsidomine treatment.

It has been shown previously that arginine treatment also improves impaired healing in diabetes. That molsidomine treatment modifies cytokine expression during wound healing and therefore improves collagen synthesis and breaking strength cannot be excluded.

In summary, administration of molsidomine can partially restore the impaired healing in diabetes by enhancing collagen synthesis and WBS. This may have therapeutic potential and needs further clinical evaluation.

Acknowledgements

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